APPLIED MICROBIOLOGY, Jan., 1967, p. 117-119
Vol. 15, No. 1

Isolation of Thermophiles from Broadleaf Tobacco and Effect of Pure Culture Inoculation on Cigar Aroma and Mildness

CLYDE F. ENGLISH, EMILY J. BELL, AND ALFRED J. BERGER[1]
Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio

Received for publication 16 August 1966

ABSTRACT

Thermophilic members of the genus *Bacillus* isolated from fermented Connecticut broadleaf tobacco included eight strains of *B. subtilis*, five strains of *B. coagulans*, four strains of *B. megaterium*, and three strains of *B. circulans*. Some of these strains in pure single or mixed culture were employed to enrich the normal thermophile flora of "sweating" tobacco. Three strains of *B. subtilis* and one of *B. circulans*, either in single or multiple enrichment, caused the more rapid appearance of a pleasing aroma in Pennsylvania "Wrapper B" filler tobacco. These conclusions are based on subjective reactions of professional testers after numerous blindfold smoking tests.

Growth of thermophilic microorganisms on "sweating" tobacco has long been known to occur, but only a few attempts to associate pure cultures with definite changes in tobacco quality have been recorded (1; D. W. McKinstry, D. E. Haley, and J. J. Reid, J. Bacteriol. **35**:71, 1938). If certain strains or species could be associated with definite desirable qualities, then the advantages to the tobacco industry would be obvious. The purpose of this investigation was to establish such associations.

MATERIALS AND METHODS

Organisms. The thermophilic strains employed in this investigation were isolated originally from "resweated" Connecticut broadleaf tobacco. Sterile swabs moistened with Trypticase Soy Broth (BBL) were used to remove organisms from the leaf surfaces and subsequently were streaked on either BBL Trypticase Soy Agar (TSA) or tobacco infusion agar (TIA) plates. Otherwise, leaf fragments were pressed onto the same media. All plates were incubated at 55 C.

TIA was prepared by allowing three or four tobacco leaves to stand in 1 liter of distilled water for 24 hr. This liquid, after being boiled for 15 min and filtered, was supplemented with TSA, 40 g per liter, and sodium chloride, 5 g per liter. The medium then was autoclaved and dispensed in 15-ml amounts into petri plates. Differential colonial morphology was used for primary selection, and all isolated cultures were maintained at 55 C or above. Diagnostic procedures were performed, and all tests, media, and reagents were used in accordance with standard microbiological methods.

Tobacco treatment. The Pennsylvania "Wrapper B" filler tobacco, which was used for the various experiments, is taken from the farms, tied into hands, gathered into 60-lb bundles, and stacked so that natural fermentation and drying processes occur for 8 to 10 months without artificial heat. Normally, at the end of this period, each bundle is moistened thoroughly by immersion in water, drained for approximately 30 min, and then placed into a sweat room for 7 to 10 days. The initial temperature is about 38 C, and the humidity is maintained at a high level so that no drying occurs. A temperature increase of an average of 5 C occurs at the center of the bundles due to microbial action. After this treatment, the tobacco is chopped into shreds, pressed into wooden cases, and placed into a second sweat room for 2 to 3 weeks. This process, "resweating," subjects the cut tobacco to an initial temperature of 42 C and a relative humidity of 85%. A temperature increase of about 10 C occurs during this stage of processing. Subsequently, the tobacco is dried and prepared for cigar manufacture.

For the various microbial tests, after the natural "dry" fermentation had been carried out and prior to the resweating procedures, the tobacco was immersed in a solution containing either a single pure culture or a mixture of pure strains. Either 250 ml or 1 liter of a 24- or 48-hr culture (pure or mixed), containing approximately 10^8 cells per milliliter and incubated at 55 C, was mixed with 50 liters of warm water for this purpose. Pure water and sterile media controls were also employed.

Cigars were prepared from the various experimentally treated tobaccos as well as from the controls. Blindfold tests by numerous smokers attested to the relative differences between the experimental and

[1] Present address: John Berger and Sons Tobacco Co., Cincinnati, Ohio.

control tobaccos. Admittedly, the results reported are based on subjective opinions; however, only those differences which appear significant are reported. Statistical consumer-market tests were not performed. It was felt that the expert opinions of professional testers would have more validity in the initial stages of the work. The tobacco experiments were conducted at the Germantown, Ohio, plant of the John Berger and Sons Tobacco Co., Cincinnati, Ohio.

RESULTS AND DISCUSSION

Twenty-two thermophilic strains were isolated and maintained at 55 C on TSA slants. This medium allowed better growth than did the TIA which, nonetheless, proved to be a good isolation medium. Twenty of the isolates proved to be aerobic endosporeforming rods conforming to the description of the genus *Bacillus*. The other two appeared to be actinomycetes and were not studied further. As a result of diagnostic procedures which included cellular and colonial morphology, of growth characteristics and rates in various media and at incubation temperatures ranging from 37 C to 65 C, and as a result of the various biochemical tests employed for the differentiation of members of this genus, the cultures were identified as thermophilic members of the following species: *B. subtilis*, eight strains; *B. coagulans*, five strains; *B. megaterium*, four strains; *B. circulans*, three strains. *B. subtilis* has been reported to be a predominant organism in a satisfactory fermentation of cigar leaf tobacco (D. W. McKinstry et al., J. Bacteriol. **35**:71, 1938).

No one strain conformed exactly to the species descriptions given in *Bergey's Manual of Determinative Bacteriology* or presented by Smith, Gordon, and Clark (4). However, the variations (biochemical) were not considered to be of such significance that another epithet should be applied. Other workers studying strains of the genus *Bacillus* have found that certain strains of various species differ from those of recognized species in being negative in one or more characters. Such cultures have been designated as intermediate (1, 3) or as weak or aberrant (4). Smith, Gordon, and Clark (4) suggested that negative characters without compensatory positive characters be considered of little diagnostic value.

In a preliminary experiment designed to test the possible effects of enriching the tobacco with specific thermophiles, nine strains were selected for study. The strains selected conformed most nearly to those isolated from tobacco by Rasper (Ch.E Thesis, Univ. Cincinnati, Cincinnati, Ohio, 1939) and included five strains of *B. subtilis*, two of *B. megaterium*, and one each of *B. circulans* and *B. coagulans*. Single additions of a 24-hr culture of each strain were made to 50 liters of water, into which a bundle (approximately 60 lb) of tobacco was immersed for several minutes. After thorough wetting, the bundles were drained for 30 min and then placed in the first sweat room. Bundles dipped into water alone and into water to which medium had been added served as controls. The subsequent treatment of the tobacco was normal, and, when it was completed, 100 cigars were made from each bundle. Blindfold tests by experts indicated that some strains had enhanced the aromatic qualtity of the tobacco. Although individual preferences were manifested, all smokers indicated that the thermophile enrichment had changed the tobacco such that it was "sweeter."

Four strains considered best in the first experiment were selected for the next experiment and included three strains of *B. subtilis* and one of *B. circulans*. The three strains of *B. subtilis* employed conformed in all respects to the species description presented in *Bergey's Manual* except for the following characteristics: strain 1 failed to produce acid from arabinose, and its optimal growth temperature was 65 C; strain $9A_2$ failed to reduce nitrates to nitrites and to produce acid from xylose and arabinose, and its optimal growth temperature was 50 C; strain 23 failed to reduce nitrates to nitrites, and its optimal growth temperature was 45 C. The strain of *B. circulans* (isolate 18) differed from the described species in that starch was not hydrolyzed and acid was not produced from xylose. The optimal growth temperature was 55 C. We used 1 liter of a 48-hr culture to enrich the water, and, to avoid as much as possible individual tobacco differences, each strain was added to three different bundles. The tobacco was cut and mixed before resweating (fermentation), and all subsequent procedures were as before. From the large amount of tobacco treated with each strain, a sample was employed to make 500 cigars for the smoking tests. There was striking consensus among the smokers that the experimental cigars were more aromatic than the controls, although there seemed to be no definite relationship between "mildness" and this aroma. All of the "expert" smokers and more than 50% of the "average" smokers (20 to 25) concurred in this opinion. Each individual smoked in a "blindfold" manner many of the cigars, intermixing the experimental with the control tobacco.

A third experiment was conducted to rule out even more so natural tobacco differences and to test a combined culture of the best strains. Eight bales were divided equally between the experimental and the control batches. It should be

pointed out that the natural sweating procedures had been in process for 5 weeks and, accordingly, even the controls were approaching normal aroma and mildness. The inoculum was decreased to 0.5 liter of a combined culture of the four strains, since the cigars in the previous smoking test would sometimes develop an "off" taste if smoked to the end. Again a definite difference towards a more aromatic tobacco was discerned by all of the test smokers.

A final experiment was run with four other types of cigar filler as well as with the Pennsylvania filler to see if combined culture application would develop more pleasing flavor in tobacco that had completed the normal fermentation and sweating procedures and had already been judged mild enough. A brief supplemental "resweating" period after culture enrichment did not reveal any significant changes in aroma or mildness.

These experiments have confirmed the observations of others (1, 2; D. W. McKinstry et al., J. Bacteriol. **35**:71, 1938) that the growth of thermophilic microorganisms is essential to the proper fermentation of cigar tobacco. They indicate further that the addition of selected thermophilic strains of the genus *Bacillus* can hasten the development of a desirable aroma and under some conditions can change and improve the smoking qualities of the tobacco. Terminal enrichment will not, however, improve the quality of tobacco which has completed the usual fermentation processes.

ACKNOWLEDGMENT

The authors are grateful to Hoke S. Greene of the University of Cincinnati, who has pioneered in this area, for his helpful suggestions.

LITERATURE CITED

1. IZQUIERDO TAMAYO, A. 1957. La flora bacteriana del tabaco. Bol. Real Soc. Espan. Hist. Nat. Secc. Biol. **10**:91–119.
2. JENSEN, C. O., AND H. P. PARMELE. 1950. Fermentation of cigar-type tobacco. Ind. Eng. Chem. (Intern. Edition) **42**:519–522.
3. KNIGHT, B. C. J. G., AND H. PROOM. 1950. A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. J. Gen. Microbiol. **4**:508–538.
4. SMITH, N. R., R. E. GORDON, AND F. E. CLARK. 1952. Aerobic spore forming bacteria. Agriculture Monograph No. 10, U.S. Department of Agriculture, Washington, D.C.